72

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(57) Abstract

A method for delivering to a target cell in a population of cells a biologically active agent comprising the steps of exposing the population of cells to a complex comprising the biologically active agent and a ligand capable of binding to the target cell and subjecting the population of cells to an electric field while maintaining cell viability.

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Transfection Process

The present invention relates to a process for the transfection of cells with targeted nucleic acid vectors. In particular, the invention describes an improved process for transfecting haematopoietic cells, especially stem cells and T-cells present in complex cell populations, with vectors targeted to specific cell-surface ligands.

The uptake of nucleic acid vectors by cells is central to molecular biology and the application of molecular biological techniques to other sciences and in clinical contexts. Since the introduction of DEAE and calcium phosphate mediated transfection techniques, it has been sought to improve the efficiency of transfection processes. This has given rise to a large number of novel protocols for delivering nucleic acid to cells, including techniques such as liposome delivery, microinjection and lipofection. One such improved technique is electroporation (see Tsong, 1991). In addition to the introduction of DNA, electroporation has also been used for the introduction of a variety of macromolecules including enzymes, antibodies and cell membrane proteins into isolated cells or tissues *in vivo* (see Weaver, 1993).

Electroporation of cells involves the application of a voltage across the cell membrane, which causes the formation of pores therein and an increase in its conductance for a short period of time, of the order of microseconds or milliseconds, so permitting the uptake of macromolecules and other substances by candidate mechanisms such as electrical drift, electrosmosis or diffusion.

Whilst the precise mechanism remains to be established, it is known that the mechanism is reversible and target cells such as erythrocytes remain viable after stable insertion of membrane proteins (Mouneimne *et al.*, 1989). Unfortunately, electroporation using the large electric fields necessary

for the introduction of nucleic acids is also associated with a high incidence of cell stress and death. The use of such large electric fields, whilst increasing the number of cells as a percentage of the starting population which take up DNA, also leads to a simultaneous decrease in the percentage of surviving cells. Therefore, the efficiency of electroporation is limited by the tendency to kill cells at the field strengths which are required to give a high transfection efficiency.

Although the general transfection techniques alluded to above, including electroporation, are effective in certain conditions, they remain ineffective when it is desired to selectively target a particular cell type in a heterogenous population of cells. This is due to the absence of any selective event in the general transfection processes. The available nucleic acid is taken up by all cells, although the efficiency of DNA uptake may vary from cell type to cell type.

Targeted uptake has been explored by a number of laboratories. One of the first effective targeted transfection techniques was disclosed by Wu (Wu and Wu, 1987; Wu *et al.*, 1989). This technique, receptor-mediated gene transfer, targeted a DNA vector to hepatocytes by complexing the vector to a glycoprotein, which is bound by asialoglycoprotein receptors on hepatocytes. Effective targeting of vectors to hepatocytes was demonstrated *in vivo* and *in vitro*.

Receptor-mediated gene transfer is dependent upon the presence of suitable ligands on the surfaces of cells which will allow specific targeting to the desired cell type followed by internalisation of the complex and expression of the DNA. This can give rise to a number of problems, since cell-surface markers are often shared between cell types. Solutions to this problem have been proposed (see, *inter alia*, UK patent application No. 9325759.0) but, to date, the

most usually cited solution is to purify the cells to be transfected, treat them and subsequently re-introduce them into the original cell population.

One solution which has been proposed which avoids the requirement to separate the target cell involves the conjugation of the vector, or delivery system, to antibodies, which can be designed to target highly specific cell-surface antigens (Wong and Huang, 1987; Roux *et al*, 1989; Trubetskoy *et al.*, 1992; Hirsch *et al.*, 1993). As in the method of Wu, nucleic acid may be attached to antibody molecules using polylysine (Wagner *et al.*, 1990; 1991).

Although the use of antibodies for vector targeting has been suggested and indeed described, the efficiency of vector uptake and expression by cells remains low (see WO 8805077, WO 9001951 and WO 9117773) and has been documented only in cell lines in culture, as opposed to human primary cells and tissues.

Increased expression of DNA derived from ligand-DNA complexes taken up by cells via an endosomal route has been achieved through the inclusion of endosomal disruption agents, such as influenza virus heamagglutinin fusogenic peptides, either in the targeting complex to in the medium immediately surrounding the target cell. However, since the mechanism of action of such peptides and their derivatives is known to be intracellular at the level of the endosome (Wiley and Skehel, 1987) their function is dependent on the presence of the entrapment of the DNA within a cellular endosome together with the peptide. Consequently, although fusogenic peptides can improve the efficiency of expression of DNA already internalised by the cell and entrapped within endosomes, the use of fusogenic peptides fails to address the issue of the efficiency of uptake into the endosomal compartment of DNA-ligand complexes bound to cell surface receptors, and hence the proportion of cells within a given cell population which are effectively transfected

whilst retaining viability.

There is a requirement, therefore, for an enhanced transfection protocol which allows targeted delivery and uptake of nucleic acid vectors to specific cells at high efficiency, preferably in the absence of purification of the cells from mixed cell populations. The enhanced protocol should therefore provide a targeted delivery system which is sufficiently specific to allow targeting of the vector to a specific cell type, yet also sufficiently efficient to ensure that a substantial proportion of the targeted cells is transfected whilst maintaining cell viability.

Such a protocol would be of immense value for therapeutic purposes, for example for the therapeutically effective delivery of nucleic acid vectors to cells of the haematopoietic lineage (see Figure 1), such as T-cells, B-cells or macrophages within a population of blood cells, or preferably to the haematopoietic stem cell itself, the progenitor of all cells within the lineage. Vectors targeted to stem cells would contain different regulatory sequences such as Locus Control Regions (LCRs) (see European Patent Application 332667) depending on whether the therapeutic benefit was to be obtained as a result of cell specific expression of the delivered gene, or whether expression was desirable in all cells through the induction of a strong ubiquitously active promoter. For example, the efficient delivery of therapeutically effective nucleic acids to haematopoietic stem cells would be of immense clinical value, for example in the correction of genetic diseases such as lysosomal storage disorders, haemophilia and haemoglobinopathies; for the modification of cells of the immune system to provide protection against pathogenic organisms such as HIV, to incite an immune response, to modulate an inflammatory or autoimmune response, or to boost the production of agents of therapeutic benefit such as calcitonin, α1-antitrypsin or a variety of growth factors or cytokines.

Clinically, there is a great advantage in targeting genes to cells of the haematopoietic lineage, since the cells can be obtained in large numbers from blood, using established procedures, transfected *ex vivo*, then replaced after transfection into the patient. This applies particularly to T-cells which represent as much as 90% of the lymphocyte sub-population of circulating white blood cells, but also to stem cells which can be mobilised in significant numbers in peripheral blood as a result of prior treatment of the patient with GCSF (see Demuynck *et al*, 1992). Stem cells are also present in cord blood and bone marrow.

Using established technology stem cells may only be used in DNA transfection after extensive enrichment procedures (See, for example, European Patent Applications 0 455 482 and 0 451 611, which disclose a method for separating stem cells from a population of haematopoietic cells). However, even current safe non-viral transfection protocols permit only a small percentage of such stem cells to be transfected.

We now describe an improved method for the delivery of biologically active agents to cells which is of great clinical benefit. It has been determined that the efficiency of receptor mediated DNA uptake protocol is enhanced by subjecting the cells to be transfected to a mild electric field in the presence of the targeting complex.

According to a first aspect of the present invention, therefore, there is provided a method for delivering to a target cell in a population of cells a biologically active agent comprising the steps of:

a) exposing the population of cells to a complex comprising the agent and a ligand capable of binding to the target cell; and

b) subjecting the population of cells to an electric

field.

Preferably, the complex further comprises an endosomal disruption agent. Advantageously, the endosomal disruption agent is of viral origin, such as a an influenza virus haemagglutinin fusogenic peptide or an analogue thereof.

It has been found that the combination of endosomal disruption agents (see Wiley and Skehel, 1987; White, 1990) and the method of the invention can result in a synergistic benefit, greatly increasing the effect of biological agents alone within host cells. It is believed, although the Applicants do not wish to limited by this theory, that the synergy is due to the operation of the method of the invention and the endosomal disruption agents at two different points in the agent internalisation process. The application of an electric field is believed to increase the internalisation of agents bound to the cell membrane via an endosomal route. The use of the endosomal disruption agent then increases the efficiency of the release of the agent from the endosome.

An advantage of the method of the invention is that unlike electroporation, which is not believed to act via an endosomal route, the viability of the target cells is maintained.

The method of the invention is suitable for targeting any cell type using an appropriate ligand, whether in a purified or heterogenous population, and displays improved efficiency of transfection and cell viability over the methods of the prior art. Accordingly, by "population of cells", it is intended to refer to purified populations consisting of a single cell type, including a single cell in isolation, as well as heterogenous populations comprising a plurality of cell types.

Preferably, the invention is applicable to unpurified,

heterogenous populations comprising a plurality of cell types, where the targeting function of the complex may be exploited to selectively target a particular cell type in the heterogenous population.

At the same time, it is possible to exploit the invention to transform more than one cell type in a mixed population of cells, by using a ligand which is specific for a common receptor.

Preferably, the target cell is a haematopoietic cell present in a purified or unpurified population of cells. Preferably, the population of cells is obtained from blood. It is an advantage of the invention that purification of the target cell from blood before treatment is not essential. Preferably, the haematopoietic target cell is a stem cell, T cell, B cell or macrophage.

The biologically active agent of the invention may be any agent capable of eliciting a biological effect in a cell. Such agents may be proteins, nucleic acids, ions or other biologically active molecules.

Preferably, the biologically active agent is a nucleic acid comprising at least one transcription unit encoding a proteinaceous or RNA molecule capable of eliciting a biological effect.

Preferably, the transcription unit encodes a protein. For example, a growth factor, hormone, cytokine, a transcription factor, a cell surface protein or a structural protein of any kind. The protein may contain one or more domains of known function, not necessarily of mammalian origin. The protein may be homologous to the target cell or deficient, absent or mutated therein. For example, the transcription unit may encode a protein effective in the therapy of an infectious disease, for example in HIV therapy. Alternatively, the transcription unit may encode a protein

capable of correcting a genetic defect or a protein deficiency.

In HIV therapy, the protein may be a non-specific toxin or an anti-viral agent whose expression or activity has been modified for anti-HIV use, for example by using an HIV-specific transactivation system to express the protein (see United Kingdom patent application No. 9305759.4). Alternatively, the agent may be specifically designed as an anti-HIV agent. For example, a decoy gene, encoding transdominant negative mutants of HIV peptides such as the tat, nef or rev gene products, which have been modified such that their activating properties are abolished but they retain the ability to compete with the natural virus-encoded protein. See WO 9014427; Echetbu and Rice, 1993; Pearson et *al.*, 1990.

For the correction of a genetic defect or a protein deficiency, such a protein may be a lysosomal enzyme for the correction of Gaisher's or Auber's disease (Scott *et al.*, 1990; Sorga *et al.*, 1987), the α or β globin gene for the correction of sickle cell anaemia or thalassaemia, or calcitonin or α1 antitrypsin to prevent the onset or progression of osteoporosis or emphysema.

Furthermore, it is envisaged that the product of the transcription unit may be an RNA molecule, such as an antisense RNA molecule (Mirabelli *et al.*, 1991) or a ribozyme tailored to act in a specific manner (Cech *et al.*, 1992).

In the present invention, a "ligand" is any entity capable of specific binding to the surface of a cell.

For example, any molecule for which a cellular receptor exists could be used as a ligand. Such substances comprise proteins, nucleic acids, carbohydrates and metal ions, optionally complexed with proteins. The use of altered

ligand molecules having engineered specificities, including a plurality of specificities, is envisaged. Especially preferred are growth factors and antibodies and antigen-binding fragments thereof, such as Fab, $F(ab')_2$ and Fv fragments. The ability of each ligand or fragment thereof to deliver a biological agent must be determined on a case by case basis. Efficiency will vary depenent on parameters such as receptor density on cells and the affinity of ligands for cell surface epitopes, as well as the precise mechanism of cell internalisation.

It should be emphasised that, in the present invention, the application of an electric field to the cells to be transfected is not equivalent to electroporation.

Unlike electroporation cell viability is maintained. The electric field applied would appear to enhance endosomal uptake, a cellular phenomenon, as opposed to electroporation which results in the production of physical pores in the cell membrane through which bodies may pass as a result of electrical drift, electrosmosis or diffusion, the proposed mechanisms for the introduction of materials using electroporation (Weaver, 1993). Furthermore, the present invention provides a selective approach for the delivery of substances to target cells within heterogenous cell populations as opposed to the indiscriminate delivery of substances using electroporation.

Preferably, the electric field used in the method of the invention, over an electrode gap of 0.4cm, operates at a capacitance of less than 600μF, advantageously less than 500μF. Most preferably, the capacitance lies between 200 and 300μF and is optimally 250μF. The voltage of the electric field is less critical to the operation of the invention, but is advantageously less than 400v and preferably between 200 and 350v.

Viability of between 60 and 100% has been obtained with the

method of the invention. In contrast, standard conditions for electroporation of peripheral blood lymphocytes are 250v at 960μF, with expected viability of about 10 to 15% (see Bio-Rad GENE PULSER® Electroprotocol for Human Primary Lymphocytes).

The method of the invention may be used *in vivo* (Powell *et al.*, 1989; Titomirov *et al.*, 1991) as well as *in vitro*. *In vitro* use is especially indicated for *ex vivo* targeted delivery of DNA to haematopoietic stem cells present in bone marrow aspirate, peripheral blood or cord blood. Transfected cells may then be returned to the patient. The avoidance of a separate cell separation step is advantageous, as handling of cells inevitably carries the risk of infection and reduced cell viability, additional patient trauma and increased healthcare costs.

According to a second aspect of the present invention, there is provided the use of a complex comprising a biologically active agent and a ligand in the preparation of a transfection mixture for use in the electrically stimulated delivery of the biologically active agent to a target cell to which the ligand is capable of binding.

The invention will now be described for the purpose of illustration only, with reference to the following figures:

Figure 1 is a schematic diagram showing the relationship of cells within the haematopoietic cell lineage;

Figure 2 shows the results of transfection of human T-cells in a total white blood cell population using an anti-CD7 antibody:DNA complex over a range of capacitance values at constant voltage (300v); the letter X indicates the expected viability of electroporated cells;

Figure 3 shows the results of the transfection of cell lines with an antibody:DNA complex in the presence or absence of

electric shock treatment and demonstrates the specificity of targeting;

Figure 4 shows the results of the transfection of primary human T-cells in a total white blood cell preparation using an anti-CD7 antibody: DNA complex (Figure 4A) and the synergistic effect of a fusogenic peptide (Figure 4B);

Figure 5 shows the reproducibility of the results shown in Figure 4;

Figure 6 shows the transfection of human peripheral blood mononuclear cells using an anti-CD33 antibody:DNA complex (Figure 6A) and an anti-MHC class II antibody:DNA complex (Figure 6B) and demonstrates synergy between electrical stimulation and the use of a fusogenic peptide; and

Figure 7 shows the results of the transfection of primary human stem cells ($CD34^+$) present in total white blood cell preparations (MNCs) from cord blood.

METHODOLOGY

Preparation of mononuclear cells
from peripheral and cord blood

An equal volume of phosphate-buffered saline (PBS) was added to a heparinized blood sample and mixed well. Carefully and gently, 10 ml of the cell suspension was layered onto 10 ml of J-PREP solution (1.077g/ml density, TechGen International) by resting the tip of the pipette against the wall of a universal tube, taking care not to disturb the surface meniscus. The preparation was centrifuged at 400 g for 30 min at room temperature. This produced a pellet of red cells and granulocytes at the bottom of the tube, a clear layer of the separation medium, a cloudy interface layer containing the mononuclear cells (MNC) and a

plasma/PBS layer on top. The interface layer was harvested using a Pasteur pipette and centrifuged at 800 g for 10 min at room temperature to collect the MNC pellet. The cells were washed twice with RPMI-5% heat inactivated fetal calf serum (RPMI/5% FCS) by centrifugating at 800 g for 5 min at room temperature.

Isolation of CD34+ cells from mononuclear cells of cord blood

Labelling of cells with biotinylated anti-CD34 monoclonal antibody

The CERATE LC system (CellPro Incorporated, WA, USA) uses an avidin-biotin immunoaffinity process to positively select CD34+ cells from a heterogenous cell population. Before starting the separation procedure primary anti-CD34 monoclonal antibody (MoAb) was removed from the freezer and allowed to thaw slowly undisturbed to room temperature (30min). The MNC were washed twice with PBS/1% bovine serum albumin (PBS/1% BSA) and resuspended in 1 ml of the same buffer. The cells were mixed with the anti-CD34 MoAb to give a final concentration of 20 µg/ml and incubated at room temperature for 20 min. After the incubation the cells were diluted to 10 ml with PBS/1% BSA and washed twice with the same buffer by spinning at 500 g for 5 min. Cells were resuspended at a concentration of 10^8/ml in PBS/5% BSA.

Preparation of the avidin column

4 ml of PBS was added to the sample chamber and the system was ensured to be air bubble-free. PBS was allowed to flow into the wash chamber. Another 5 ml of PBS was added to the sample chamber and the wash chamber topped up to 10 ml with PBS. Slowly, the pre-gel was added dropwise to sample chamber (a uniform 0.7 ml bed of pre-gel formed on the bottom of sample chamber above the avidin column which serves to trap tissue debris and cell clumps). 5 ml of PBS

was allowed to pass through. 5 ml of PBS/5% BSA solution was added and the flow stopped when the buffer level reached the top of the pre-gel.

Cell separation protocol

Antibody-labelled cells were layered onto pre-gel in sample chamber (4 ml maximum) and eluted unabsorbed cells collected in a fresh tube. When cell sample reached the top of the pre-gel, an additional 2 ml of PBS/5% BSA was added to flush any remaining cells into the avidin column. When the remaining buffer reached the top of pre-gel PBS was allowed to flow between the wash chamber and the avidin column (making sure not to allow air into avidin column at this stage). When the PBS in the wash chamber reached 5 ml mark the flow was stopped. A fresh tube containing 1 ml PBS/5% BSA was placed to collect absorbed cells. Flow was started and the avidin column squeezed vigorously 3-5 times. 1 ml PBS was allowed to flow into the collecting tube. The squeeze was repeated and left to flow until all of the PBS was drained from wash chamber. The collected cells were washed twice with RPMI/5% FCS and resuspended in 2 ml of the same buffer before counting the cells. The cells were placed in a 95% air 5% CO_2 incubator for 30 min before performing a FACS analysis of CD34+ cells in the collected fraction using a different anti-CD34 MoAb.

<u>Transfection Procedure</u>

Preparation of monoclonal antibodies-polylysine conjugates

Monoclonal antibodies to various human haematopoietic cell surface markers were conjugated to poly(L-lysine)(MoAb-PL) with a 196-278 chain length. Ligand:polylysine:DNA complexes were constructed following the procedures first described by Wu and Wu (1987) as subsequently modified by Birnstiel and coworkers (Wagner *et al.*, 1990; 1991). The ratio of ligand:polylysine and peptide:polylysine per DNA

targeting complex can be determined by titration on a cell type to cell type basis.

Antibody-polylysine conjugates were synthesised through disulphide linkages, which were formed using the bifunctional reagent 3-(2-Pyridylilthio) propionic acid N-hydroxysuccinimide ester (SPDP: Sigma Chemical Co.) using a modification of the protocol described by Wagner E. *et al.*, Proc. Natl. Acad. Sci. U.S.A., 87, 3410-3414, 1990. Antibodies were purified prior to conjugation and transferred into phosphate or N-(2-hydroxyethyl)piperazide N'-(2-hydroxyethyl)piperazine N'-2-(ethanesulfonic acid), HEPES buffered saline solutions. Poly-L-lysine hydrobromide salts with an average chain length of 200 monomer units and a range of 117-234 units were obtained from Sigma Chemical Co.

SPDP modification of antibody

SPDP, solubilized in dried acetonitrile at a concentration of 20mM was added to antibodies at a molar ratio of 10:1, the antibodies being in either HBS (pH 7.9) or PBS (pH 7.5) solutions at concentrations of 1-5 mg/ml. The reactants were mixed at room temperature for 1 hr, after which the excess and free linker acid released during the antibody labelling were removed by size exclusion chromatography (G25: Pharmacia), the collected labelled antibody was then stored at +4°C. The pyridine-2-thione content of labelled antibody was determined by measuring the increase in 343 nm absorbence in the presence of Dithiothreitol (DTT), antibody concentration was determined by A280 measurement, (correcting for the effect of pyridine-2-thione content, Carlsson, J. *et al.*, Biochem. J., 173,723, 1978) using an extinction coefficient of 1.7 Au 280 ml/mg antibody. The level of linker loading was then determined by relating the pyridine-2-thione content to the antibody content. Typically, ratios of 2-6 linker units per antibody were obtained.

Modification of poly-L-Lysine

Polylysine solubilized in 50 mM Hepes pH 7.9 at a concentration of 20 mg/ml, was reacted with SPDP (prepared as previously described), the SPDP being added at a 5:1 molar excess. The reactants were mixed at RT for 1 hr, after which the solution pH was adjusted to 5 by the addition of 1M Sodium acetate pH 5.0.

Excess SPDP and free linker acid were removed by size exclusion chromatography (G25: Pharmacia equilibrated in 20mM sodium acetate buffer pH 5.0) and the pyridine-2-thione content of the polylysine determined as previously described. This value was related to the original quantity of polylysine added to the reaction mixture to give a value for the linker loading on the polylysine. Typically values between 1-4 were obtained. The pH of the collected polylysine pool was adjusted to 7.9 by the addition of 1M Hepes buffer pH 7.9, and stored at +4°C.

The labelled polylysine solution was reduced by the addition of a 10-15 molar excess of DTT and reacted for 30-60 min's at RT in order to produce free thiol groupings. The pH of the solution was then adjusted to 5.0 by the addition of 1M Sodium acetate pH 5.0 and excess DTT removed by size exclusion chromatography (G25: Pharmacia). The free thiol levels of the polylysine pools were determined using 5,5'-Dithio-bis(2Nitrobenzoic acid) Ellman's reagent (Deakin H. *et al.*, Biochem. J., 89, 296, 1963).

Conjugation of antibody with polylysine

Labelled antibody was diluted to a level of <= 1mg/ml with 50 mM Hepes pH 7.9 and glycerol added to 20% v/v. Labelled Polylysine was added at a 5:1 excess to labelled antibody and the reactants mixed for 16-20 hrs. at 10°C (the antibody/polylysine ratios being based on measured antibody

concentrations, and assumed polylysine concentration). The conjugation levels achieved were determined by measuring the 343 nm absorbence and comparing to that in the original solution. The increase in 343 nm absorbence was then related to the original pyridine-2-thione content, and the amount of free thiol added to the original conjugation mixture. Typically the results showed that 50-100% of the available dipyridyl groups were reacted and that 10-50% of the free thiol added were reacted in the various conjugation mixtures, suggesting polylysine to antibody levels of between 2 and 5.

Purification of conjugates

Conjugates were purified by cation exchange chromatography (Fractogel EMD SO_3^- E. Merck). Prior to purification NaCl was added to the conjugate mixtures to a level of 0.6 M by the addition of 3M NaCl 50 mM Hepes pH 7.9. The chromatography columns were equilibrated in 0.6 M NaCl 50 mM Hepes pH 7.9 prior to sample loading and a 0.6-3.0 M NaCl gradient was run over the column in order to elute the conjugated material, conjugate being eluted been 1.0 and 2.5 M NaCl.

The presence of conjugate in the eluted material was determined by SDS PAGE, in which samples were loaded in reduced and non-reduced states. Conjugates were defined as samples which did not enter the gel under non reducing conditions but showed normal antibody profiles under reducing conditions.

The binding specificity of all antibody-polylysine conjugates was then compared with antibody alone by FACS analysis to ensure that chemical modification had not diminished or altered the binding specificity of the antibody polylysine conjugates.

Preparation of antibody-polylysine:DNA complexes

RSVLuc, a DNA plasmid containing the luciferase gene under control of the RSV promoter (provided by Dr. M. Cross), as a reporter gene were used. The appropriate amount of DNA was diluted in HBS (120 mM NaCl, 20 mM Hepes, pH 7.4) and the MoAb-PL solution added in HBS dropwise with constant mixing. The complex mixture was allowed to stand at least for 30 min at room temperature. The amount of DNA that bound to different MoAb-PL preparations differed according to the binding characteristics of each preparation (the mass ratio of MoAb-PL to DNA ranges from 0.5:1 to 2:1). However, the DNA concentration in the complex should not exceed 20 µg/ml otherwise precipitation of the complex will occur. In a typical experiment the complex was formed by mixing 300 µl HBS solution containing 5 µg of RSVLuc with 100 µl of HBS solution containing 10 µg of HB2-PL278 (anti-CD7).

Incubation conditions of haematopoietic cells with the monoclonal antibody-polylysine/DNA complex and the Influenza virus haemagglutinin HA-2 N-terminal fusogenic peptide

5×10^6 cells were resuspended in 3 ml of RPMI/5% FCS supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The MoAb-PL/DNA complex solution was added at 5-10 µg MoAb/10^6 cells (2 ml of the HB2-PL/DNA complex solution in the case of the typical experiment cited above). An influenza fusogenic peptide (GLFGAIAGFIGAGTGGMIAGGGC; synthesised by Neosystem, Strasbourg, France) was added at a final concentration of 30 µM. The tube containing the cell suspension was gassed with 5% CO_2, the cap of the tube tightened, and the tube left to stand on wet ice for 2h with gentle shaking every 30 min to allow for saturation of all the relevant cell surface marker with the antibody complex to occur. The tubes were transferred to a 5% CO_2 incubator and left for 45 min to allow for internalization and cycling of the MoAb-PL/DNA.

Electric Shock Procedure

At the end of incubation the cells were washed once with RPMI/5% FCS and once with ice cold PBS by spinning at 800 g for 10 min to remove non-bound MoAb-PL/DNA complex. The cells were resuspended in 0.5 ml of electroporation-type Hepes buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 5 mM $MgCl_2$, 25 mM Hepes, pH 7.4) (pipetting the cells should be avoided at this stage; they were easily resuspended by carefully swirling the tubes). The cell suspension was transferred into a 4 mm interelectrode distance sterile electroporation cuvette. The cuvettes containing the cell suspensions were kept on ice for approximately 2 min. Before electric shock treatment the outside surface of the cuvettes was dried thoroughly to avoid a short circuit. A single voltage pulse was applied at the conditions of 300 V and 250 μF capacitance using a Gene-Pulser system (Bio-rad). After the electric shock treatment the cells were cooled on ice for 2 min then carefully removed using a sterile plastic Pasteur pipette into a fresh tube. The cells were diluted 10X (5 ml total) with RPMI/5% FCS. Cells were collected by low speed centrifugation (150 for 4 min). The cells were resuspended in 3 ml RPMI/40% FCS, placed in a 12 well culture plate and incubated for 24h before assessing the luciferase activity. Cell viability was determined by dye exclusion.

Example 1

Human primary T-cells were obtained from human blood and subjected to transfection as described with an anti-CD7 antibody:RSVLuc complex according to the method of the invention, in the absence of fusogenic peptide, at a variety of capacitance values on the Bio-Rad gene pulser apparatus. At optimum levels, it can be seen in Figure 2 that the viability of cells remains essentially unchanged when compared to the control at 0μF (i.e. no electric field).

Example 2

CEM cells carrying the CD7 surface marker were transfected with RSVLuc complexed to anti-CD7 and anti-CD19 antibodies, according to the protocol described above. The results are shown in Figure 3.

As a control, uncomplexed DNA was used to transfect CEM cells in the presence or absence of the electric shock treatment. Luciferase gene activity in the control experiment was very low and, significantly, was not affected by the electric shock treatment. This shows that the electric field used in the present method does not give rise to effective electroporation of the cell membrane.

Likewise, no effect was seen when the electric shock treatment was applied to cells transfected with DNA complexed to anti-CD19. CEM cells are CD 19$^-$.

However, a large increase in luciferase gene activity is seen with cells transformed with anti-CD7 complexed DNA upon administration of the electric shock.

Example 3

White blood cells were isolated from whole human blood by centrifugation. The T-cells present in the crude white blood cell preparation were targeted with an anti-CD7: RSVLuc complex, optionally further comprising an influenza virus haemagglutinin fusogenic peptide.

The results, shown in Figure 4, demonstrate that, in the presence of the electric shock treatment of the invention, vastly superior transfection efficiency is observed. The use of a fusogenic peptide further enhances the efficiency obtainable by the method of the invention.

Example 4

The experiment described in Example 3 was repeated five times in order to demonstrate the reproducibility of the results. The data from the five experiments appear in Figure 5.

Example 5

The experiments described in Example 3 were repeated except that white blood cells (PBMNCs) isolated from whole human blood were targeted with an anti-CD33:RSVLuc complex (Figure 6A) or an anti-MHC class II:RSVLuc complex (Figure 6B). The results demonstrate the synergistic effect between fusogenic peptide and electric shock treatment in the enhancement of transfection efficiency while maintaining cell viability.

Example 6

Stem cells in a total white blood cell population (MNCs) isolated from whole human cord blood were targeted with QBEND-10, an anti-CD34 antibody, complexed with RSVLuc.

Cells were not fractionated and contained 3.4% CD34+ cells. The effect of the use of electric shock treatment and a fusogenic peptide was assayed. The results are shown in Figure 7 and demonstrate the enhancement of transfection by the use of an electric field and fusogenic peptide over fusogenic peptide alone.

Example 7

To demonstrate the specificity of anti-CD34:DNA complex delivery to stem cells in total white blood cell preparations, white blood cells were isolated from human blood and 2.5×10^7 mononuclear cells incubated with the complexes shown in table 1 for 2 hours at 4°C. The cells were then washed twice for 45 minutes at 37°C and subjected

to electric shock treatment (except sample D).

Viability was determined and is shown in table 1. It should be noted that the results should be compared to the level for sample D, which was not subjected to the procedure of the invention.

After reaction at 37°C for 24 hours, cells were fractionated on CD34 cell selection flasks and counted.

Luciferase activity was then assayed in the cells, with the results shown in table 1. It can be seen that luciferase activity is only detectable over background levels in $CD34^+$ cells, demonstrating the specificity of the targeting system used.

The invention is described above by way of example only, and numerous modifications of detail will be apparent to those skilled in the art which fall within the scope of the appended claims.

Table 1

	Viability		Cells Recovered		Luciferase Activity L.U./mg		Electric	Fusogenic
Sample	total	relative	$CD34^+$	$CD34^-$	$CD34^+$	$CD34^-$	Shock	Peptide
A	70%	83.3%	6.3×10^5	1.35×10^7	1457	1800	+	-
B	60%	71.4%	5.6×10^5	1.20×10^7	2800	1500	+	-
C	52%	61.9%	4.8×10^5	1.00×10^7	4428	1642	+	+
D	84%	100%	4.9×10^5	1.15×10^7	1814	1756	-	-

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Claims:

1. A method for delivering to a target cell in a population of cells a biologically active agent comprising the steps of:

a) exposing the population of cells to a complex comprising the biologically active agent and a ligand capable of binding to the target cell; and

b) subjecting the population of cells to an electric field.

2. The method of claim 1 wherein the complex comprises an endosomal disruption agent.

3. The method of claim 2 wherein the endosomal disruption agent is an influenza virus haemagglutinin fusogenic peptide or an analogue thereof.

4. The method of any preceding claim wherein the population of cells is heterogenous.

5. The method of any preceding claim wherein the ligand is specific for the target cell.

6. The method of any preceding claim wherein the biologically active agent is a nucleic acid molecule.

7. The method of claim 6 wherein the nucleic acid molecule encodes a protein or ribozyme.

8. The method of any preceding claim wherein the biologically active agent is useful in therapy.

9. The method of claim 8 wherein the biologically active agent is useful in the correction of a genetic defect.

10. The method of claim 8 wherein the biologically active agent is useful in the therapy of an infectious disease.

11. The method of claim 8 wherein the biologically active agent is useful in the correction of a protein deficiency.

12. The method of claim 10 wherein the infectious disease is AIDS.

13. The method of claim 12 wherein the biologically active agent is a decoy protein or ribozyme.

14. The method of any preceding claim wherein the ligand is an antibody or a fragment thereof.

15. The method of any one of claims 1 to 14 wherein the ligand is a growth factor or a fragment thereof.

16. The method of any preceding claim which is used *in vivo*.

17. The method of any preceding claim wherein the target cell is a stem cell.

18. The method of claim 17 wherein the target cell is a haematopoietic stem cell.

19. The method of any one of claims 1 to 16 wherein the target cell is derived from hematopoietic stem cells.

20. The method of claim 19 wherein the target cell is a T cell.

21. A method for the treatment of a disease comprising removing cells from the body of a patient and treating them according to the method of any one of claims 1 to 8, before reintroducing them into the body of the patient.

22. The use of a complex comprising a biologically active agent and a ligand in the preparation of a transfection mixture for use in the electrically stimulated delivery of the biologically active agent to a target cell to which the ligand is capable of binding.

23. A method or use as hereinbefore described, with reference to the examples.

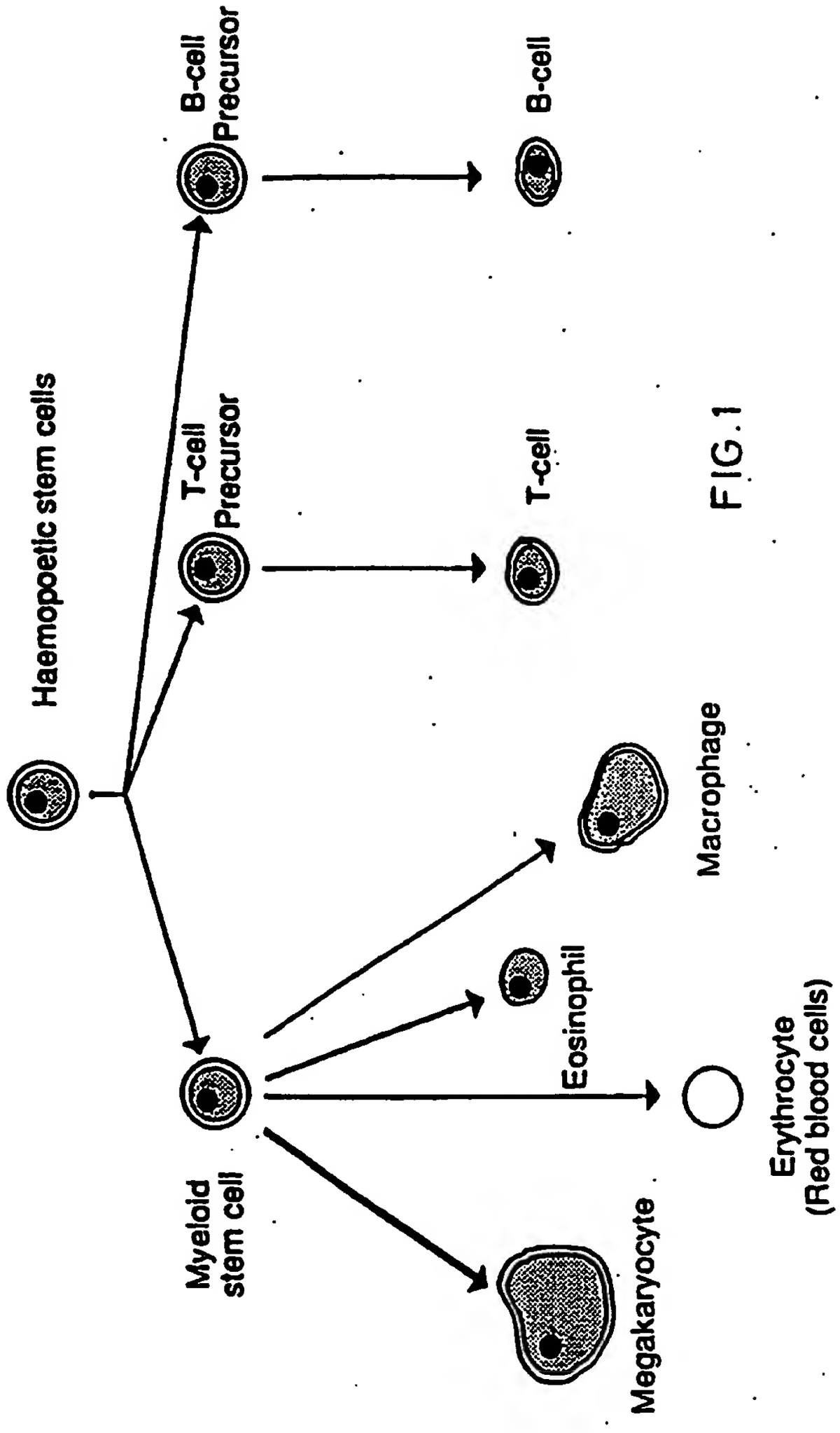

Haemopoetic stem cells
B-cell Precursor
B-cell
T-cell Precursor
T-cell
Macrophage
Eosinophil
Erythrocyte (Red blood cells)
Myeloid stem cell
Megakaryocyte

FIG.1

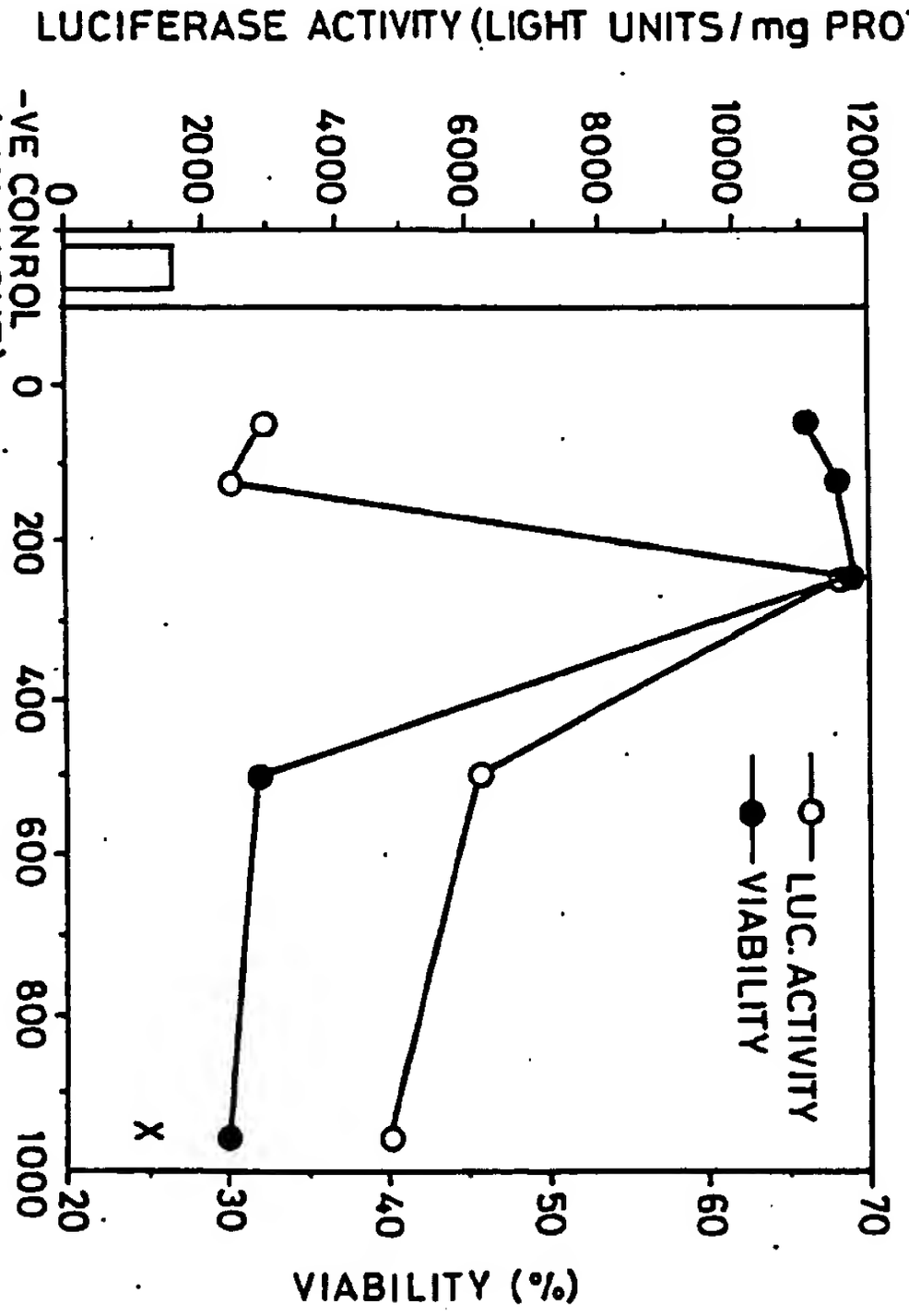

LUCIFERASE ACTIVITY (LIGHT UNITS / mg PROTEIN)
12000
10000
8000
6000
4000
2000
0
-VE CONROL (DNA ALONE)
0
200
400
600
800
1000
CAPACITANCE (μF)
LUC. ACTIVITY
VIABILITY
X
20
30
40
50
60
70
VIABILITY (%)

FIG. 2

FIG. 3

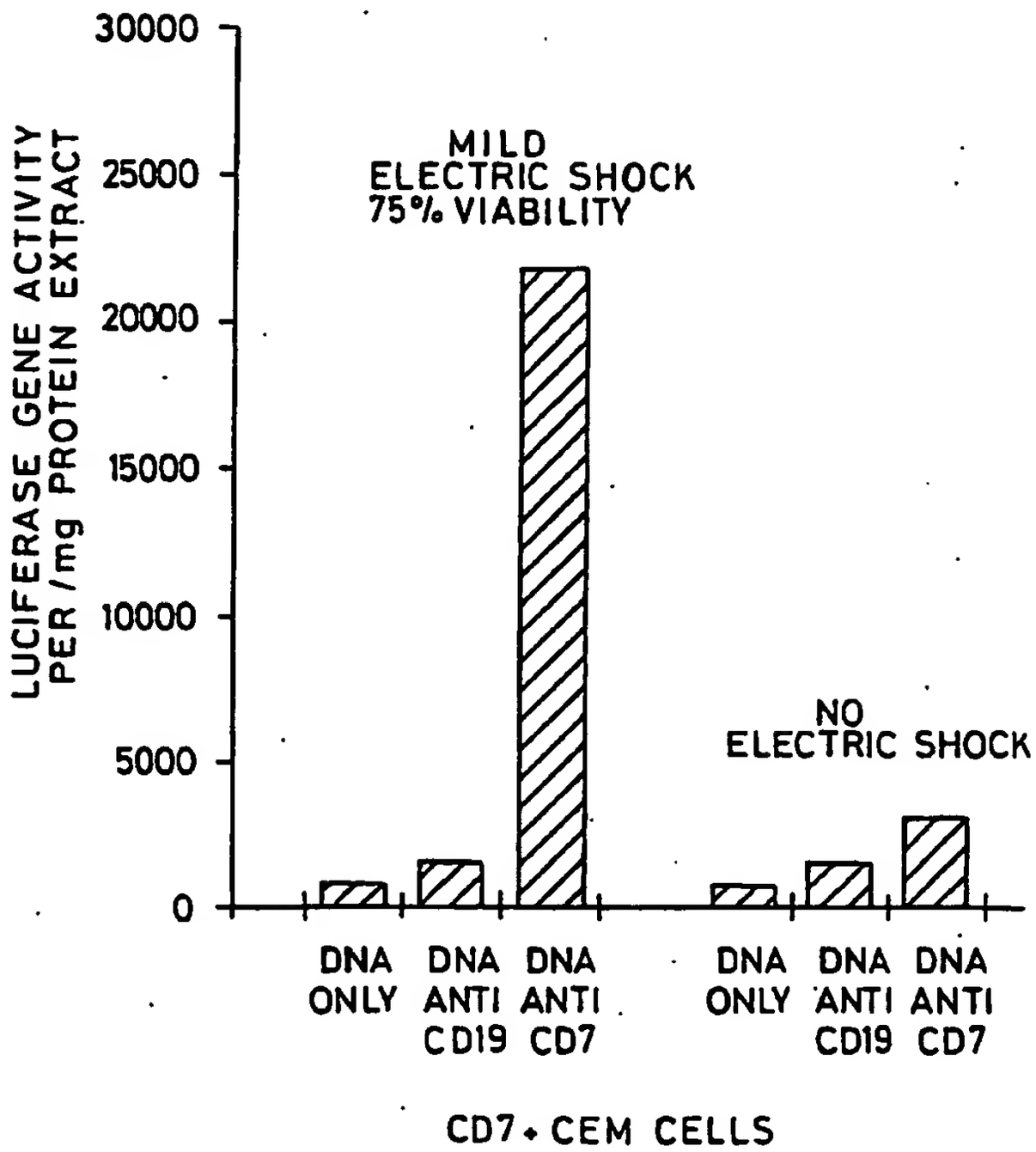

LUCIFERASE GENE ACTIVITY PER /mg PROTEIN EXTRACT
30000
25000
20000
15000
10000
5000
0
MILD ELECTRIC SHOCK 75% VIABILITY
NO ELECTRIC SHOCK
DNA ONLY
DNA ANTI CD19
DNA ANTI CD7
DNA ONLY
DNA ANTI CD19
DNA ANTI CD7
CD7+ CEM CELLS

FIG. 4B

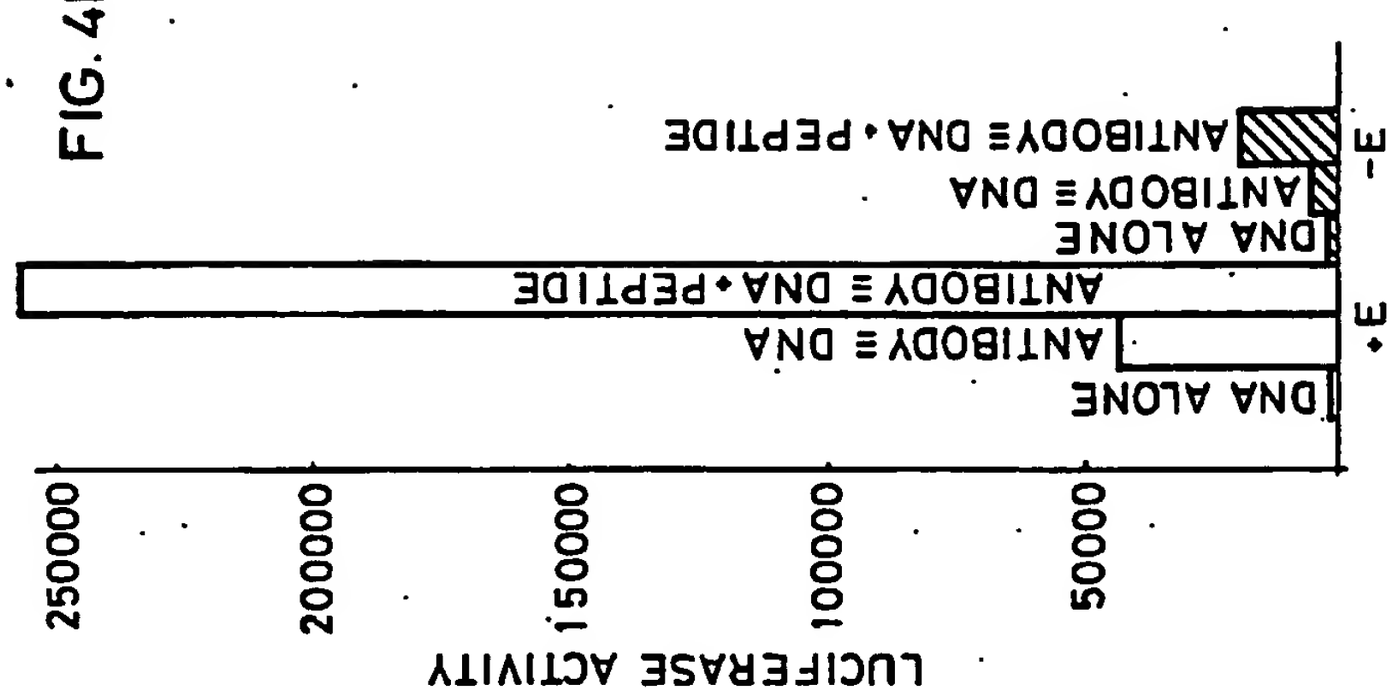

DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA • PEPTIDE
-E
+E
DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA • PEPTIDE
+E
50000
100000
150000
200000
250000
LUCIFERASE ACTIVITY

FIG. 4A

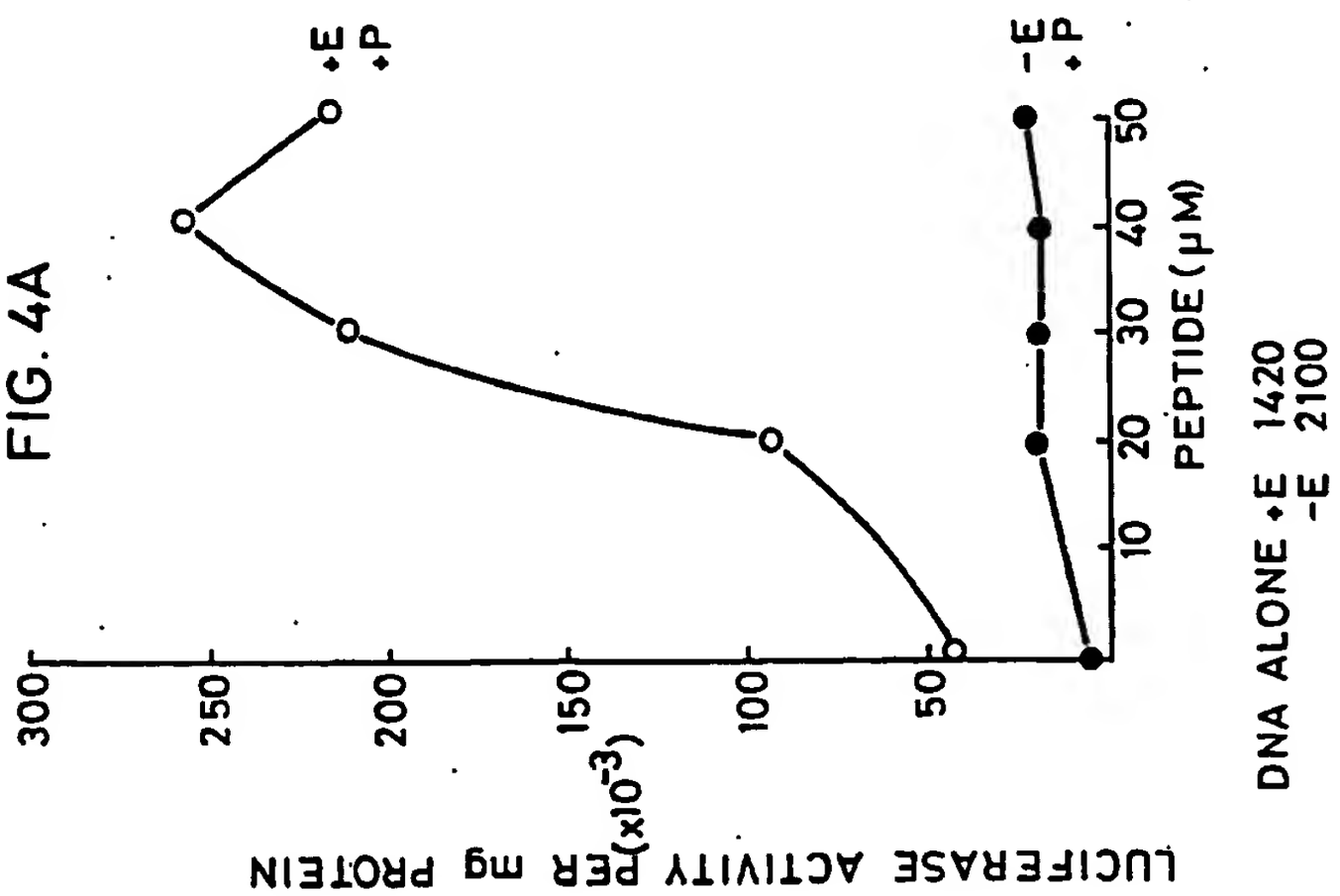

+E +P
-E +P
10
20
30
40
50
PEPTIDE (μM)
DNA ALONE +E 1420
-E 2100
50
100
150
200
250
300
LUCIFERASE ACTIVITY PER mg PROTEIN (x10⁻³)

FIG.7

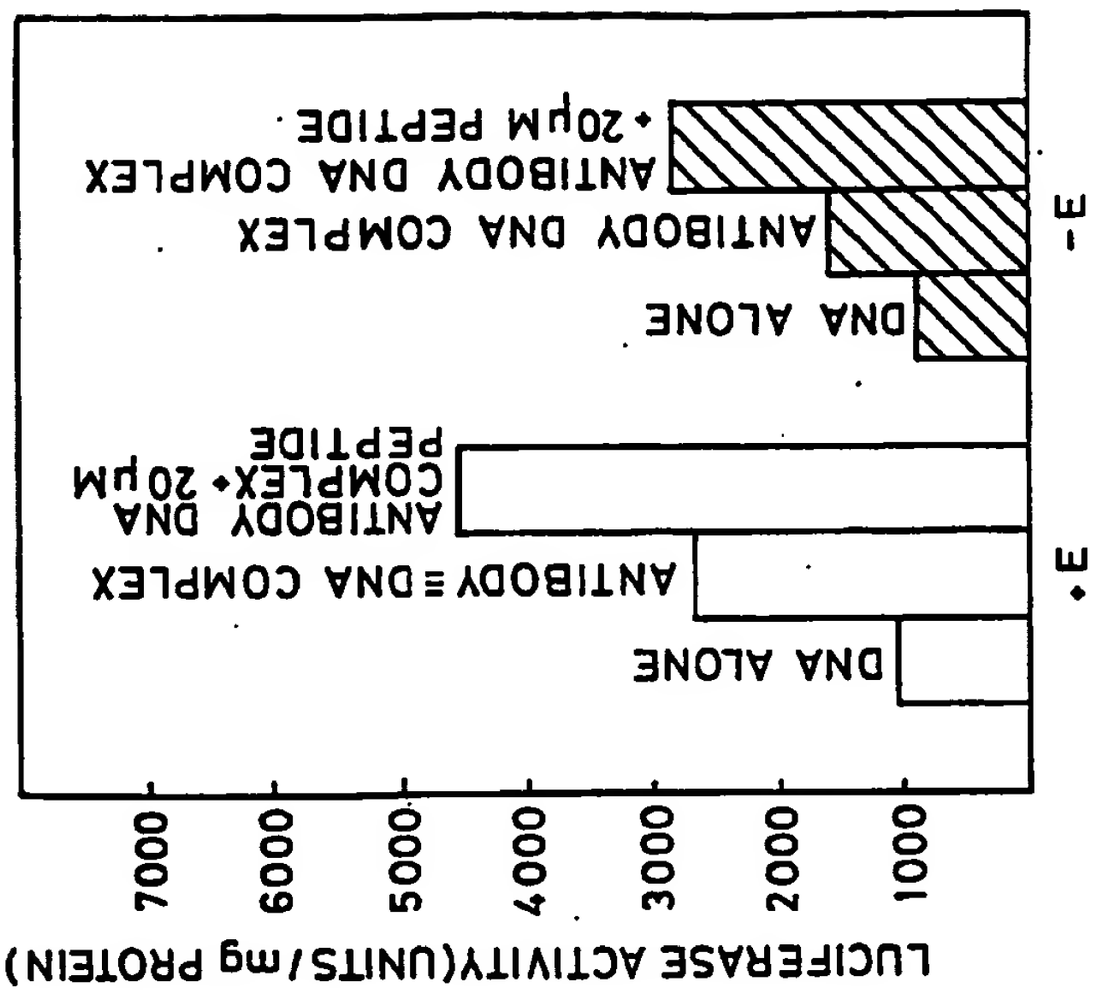
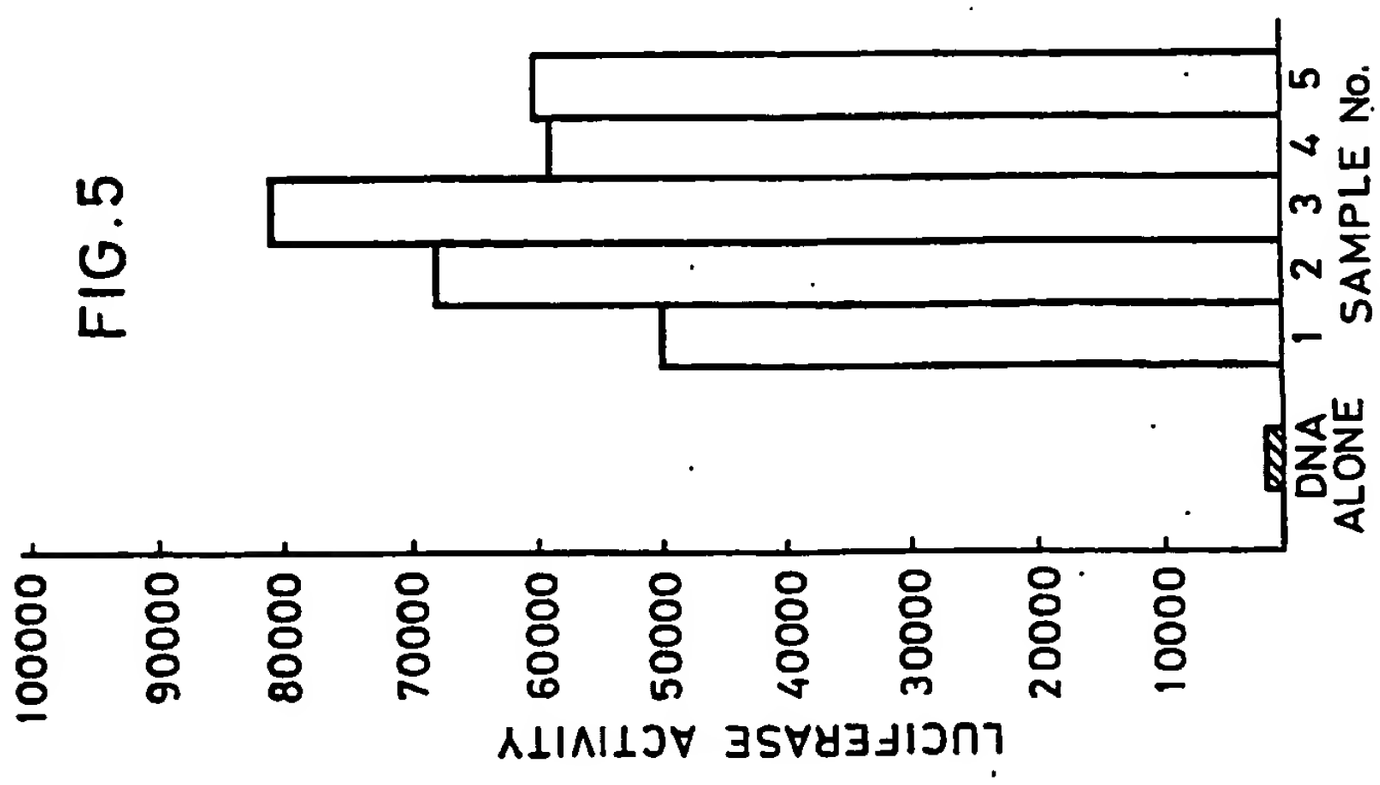

DNA ALONE
ANTIBODY ≡ DNA COMPLEX
ANTIBODY DNA COMPLEX • 20μM PEPTIDE
E•
DNA ALONE
ANTIBODY DNA COMPLEX
ANTIBODY DNA COMPLEX • 20μM PEPTIDE
E•
7000
6000
5000
4000
3000
2000
1000
LUCIFERASE ACTIVITY (UNITS / mg PROTEIN)

FIG.5


1 2 3 4 5
SAMPLE No.
DNA ALONE
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
LUCIFERASE ACTIVITY

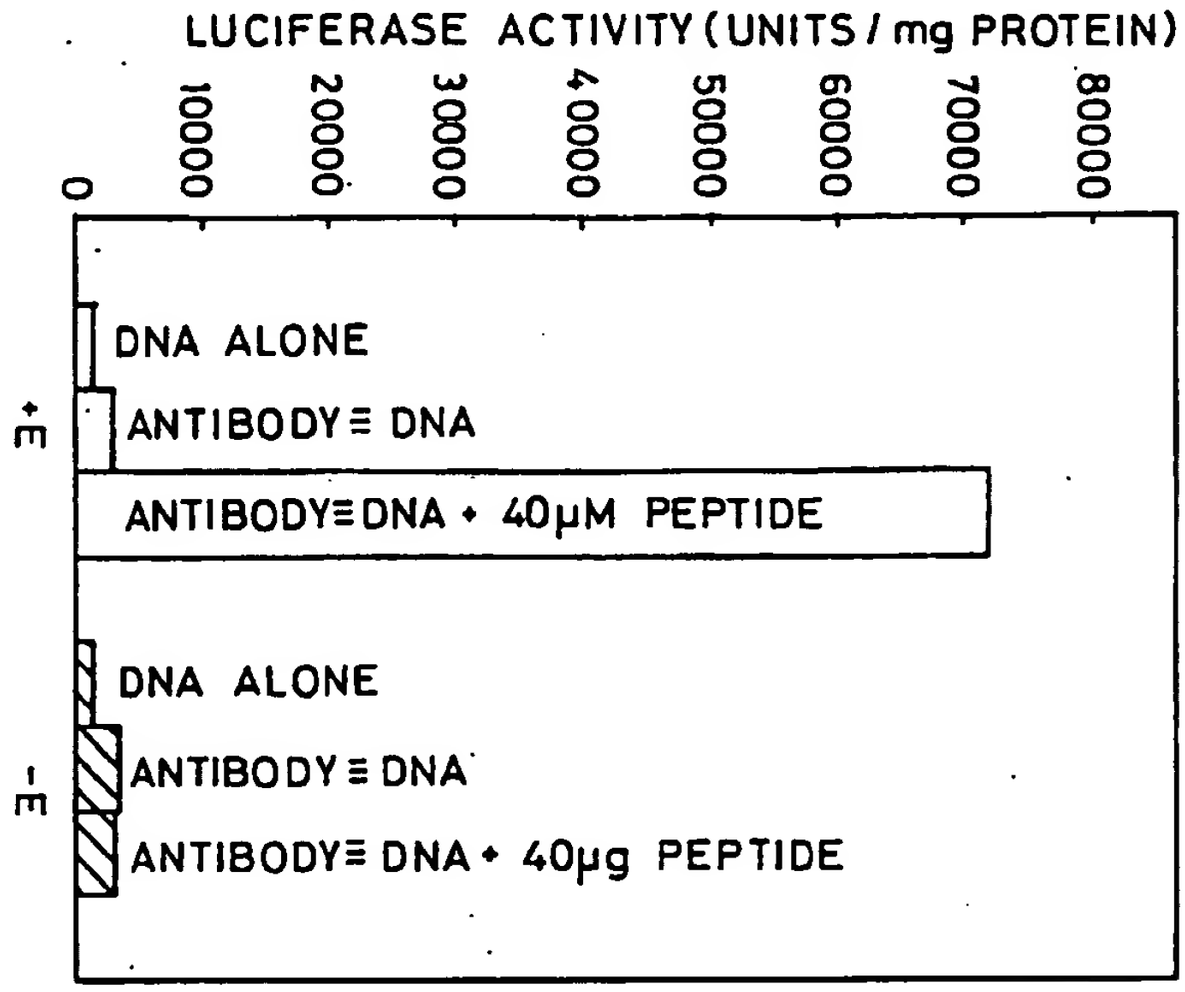

LUCIFERASE ACTIVITY (UNITS / mg PROTEIN)
0
10000
20000
30000
40000
50000
60000
70000
80000
E+
DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA + 40µM PEPTIDE
E-
DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA + 40µg PEPTIDE

FIG.6A

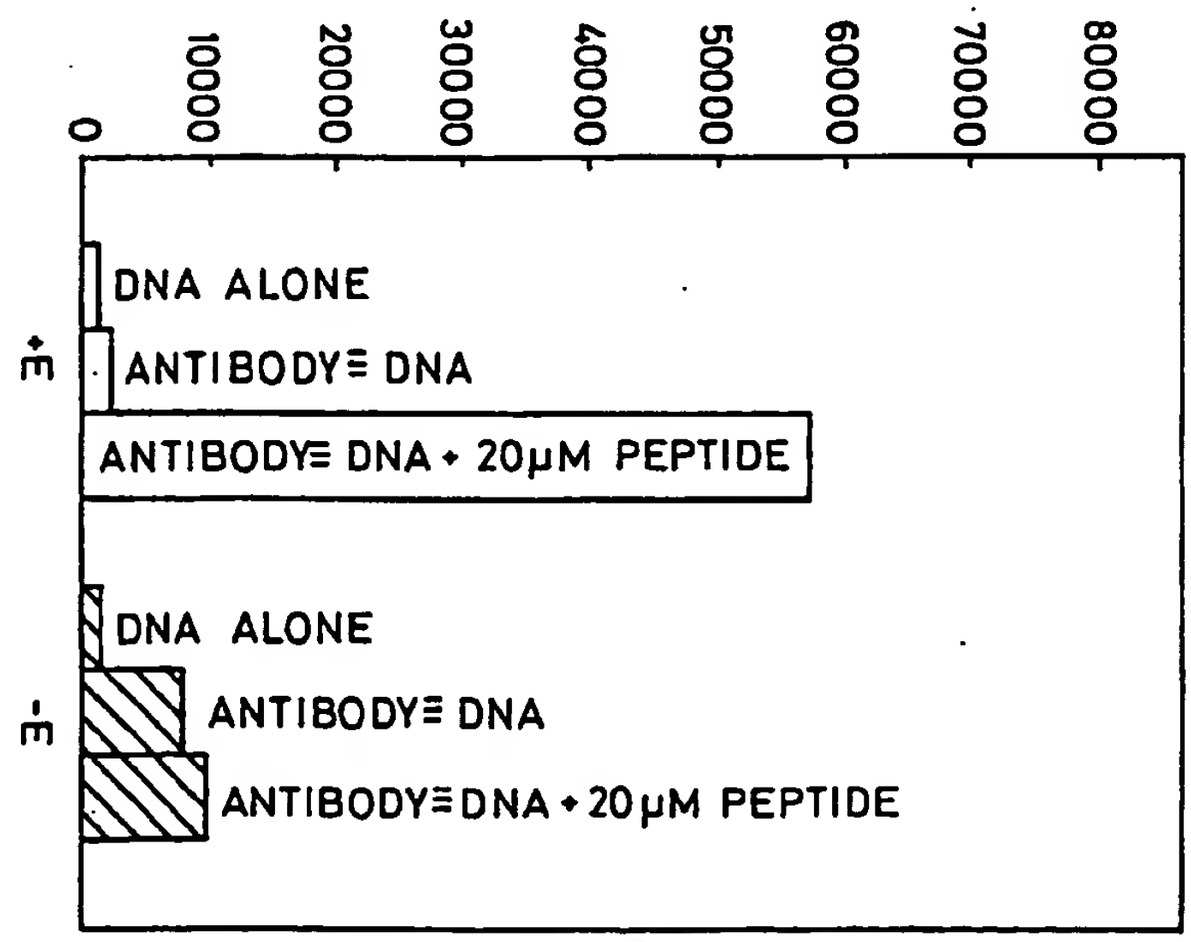

0
10000
20000
30000
40000
50000
60000
70000
80000
E+
DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA + 20µM PEPTIDE
E-
DNA ALONE
ANTIBODY ≡ DNA
ANTIBODY ≡ DNA + 20µM PEPTIDE

FIG.6B